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TITLE: Integration of Genomic, Biologic, and Chemical Approaches to Target p53 Loss and Gain-of-Function in Triple Negative Breast Cancer

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14. ABSTRACT

This is the second annual progress report for DoD Award W81XWH-13-1-0287 / BC123219, investigating biochemical states resulting from alterations in the p53 signaling pathway in triple negative breast cancer (TNBC). Development of therapies for TNBC is a clinical and scientific challenge due to the heterogeneity of the disease and the lack of recurrent, drug-targetable molecular alterations. Our research focuses on the p53 tumor suppressor pathway, which is altered in the majority of TNBC cases and produces two adaptive states: loss of function (LOF) of wild-type p53 through mutation, gene silencing, or amplification of negative p53 regulators, and gain of function (GOF) displayed by some "hotspot" p53 mutant proteins that accumulate to high levels within the cell and drive oncogenic phenotypes including growth, migration, and drug resistance. We hypothesize that targeting these adaptive biochemical states will provide candidate therapeutic targets for a large fraction of TNBC, a cancer for which there are no molecular targets to date. We are pursuing two specific aims: 1) to identify which signaling pathways, in either adaptive state, are required for TNBC cell viability, and 2) to test validated targets for "druggability" by fragment-based screening and develop small molecular inhibitors against targets that are both valid and druggable.

15. SUBJECT TERMS

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1. INTRODUCTION

This is our second annual progress report for DoD Award W81XWH-13-1-0287, investigating biochemical states resulting from alterations in the p53 signaling pathway in triple-negative breast cancer (TNBC). Development of therapies for TNBC is a clinical and scientific challenge due to the heterogeneity of the disease and the lack of recurrent, drug-targetable molecular alterations[1-3]. Our research focuses on the p53 tumor suppressor pathway, which is altered in a majority of TNBC cases and produces two adaptive states: loss of function (LOF) of wild-type p53 through mutation, gene silencing, or amplification of negative p53 regulators, and gain of function (GOF) displayed by some "hotspot" p53 mutant proteins that accumulate to high levels within the cell and drive oncogenic phenotypes including growth, migration, and drug resistance[4-7]. Our continuing working hypothesis is that targeting these adaptive biochemical states will provide candidate therapeutic targets for a large fraction of TNBC, a cancer for which there are no molecular targets to date. We are pursuing two specific aims: 1) to identify which signaling pathways, in either adaptive state, are required for TNBC cell viability, and 2) to test validated targets for "druggability" by fragment-based screening and develop small molecular inhibitors against targets that are both valid and druggable.

2. KEYWORDS

Listed in original application with emphasis on the following in this renewal:

p53
triple-negative breast cancer
subtypes
gene expression
somatic cell genetics
CRISPR/Cas

3. ACCOMPLISHMENTS

Major Goals of Project and Accomplishments

Specific Aim 1: We will verify which clinically characterized p53 mutants are oncogenic in TNBC lines and model systems. TNBC cell lines representative of the different adaptive states will be subjected to high-throughput siRNA-based synthetic lethality screens as a primary search for signaling pathways that, when targeted, can impact viability under a given adaptive state. One sub-aim is to target the p53 LOF adaptive state, and the other is to identify pathways that provide insight to how select high frequency gain of function p53 mutants can confer an oncogenic state. The intent is to identify key pathway components that can be advanced as candidate targets for "druggability."

In the last annual report, we described our progress toward the creation of an isogenic p53 mutant TNBC cell line panel using CRISPR/Cas-mediated genome editing[8]. In the present reporting period, we have continued the creation and characterization of this panel and have engineered over 16 isogenic clones expressing three of the highest-frequency p53 GOF mutants found in TNBC, along with wild-type and LOF controls. Functional validation of a subset of these clones is presented in Figure 1. These isogenic p53 mutant-expressing cell lines will serve as valuable cell context-identical reagents for biochemical and transcriptional assays to identify targets of interest in the p53 mutant GOF and LOF states.

In our preliminary characterization of the isogenic mutant panel, we compared the half-maximal inhibitory concentration (IC_{50}) of several standard-of-care chemotherapeutic agents used in TNBC

along with Nutlin-3, an MDM2/p53 binding inhibitor that targets cancer cells expressing wild-type p53. As we hypothesized, only the wild-type parental cell line was sensitive to Nutlin; each of the isogenic cell lines engineered to express GOF or LOF p53 mutants was comparatively insensitive (Figure 2A).

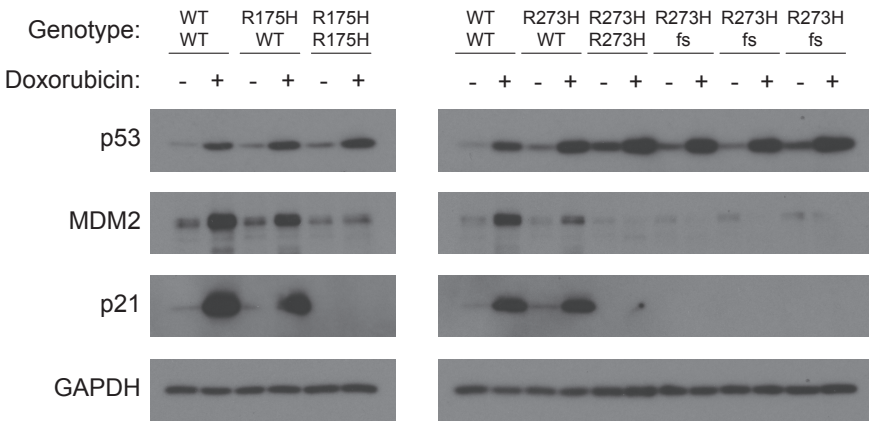

Figure 1. CRISPR/Cas-mediated *in vitro* somatic cell recombination produces isogenic p53 mutants in TNBC cell lines. A diploid, p53 wild-type TNBC cell line (+/+) was targeted with a plasmid encoding *S. pyogenes*-derived Cas9 protein[9] and homology-directed repair templates for the indicated missense mutants. The resulting single cell colonies were sequenced and identified to contain a variety of heterozygous and homozygous mutants, as indicated at the top of the figure. Each cell line was incubated +/- 0.2 μM doxorubicin as indicated, and immunoblotting was performed for p53 or its transcriptional targets MDM2 and p21. GAPDH is included as a loading control.

Intriguingly, when we evaluated the relative sensitivities of the cell lines to doxorobucin and paclitaxel, two agents used as first-line therapies for TNBC patients, there were clear differences between the R175H and R273H p53 GOF mutants (Figure 2B-C). This is in agreement with previous reports documenting chemo-resistance as a GOF activity of specific p53 mutants in the setting of ectopic overexpression[9]; however, our isogenic cell line panel should provide a significant advantage in investigating the biochemical basis of this chemoresistant GOF. At present, we are expanding the characterization of sensitivity to a wider panel of established and investigational pharmaceuticals.

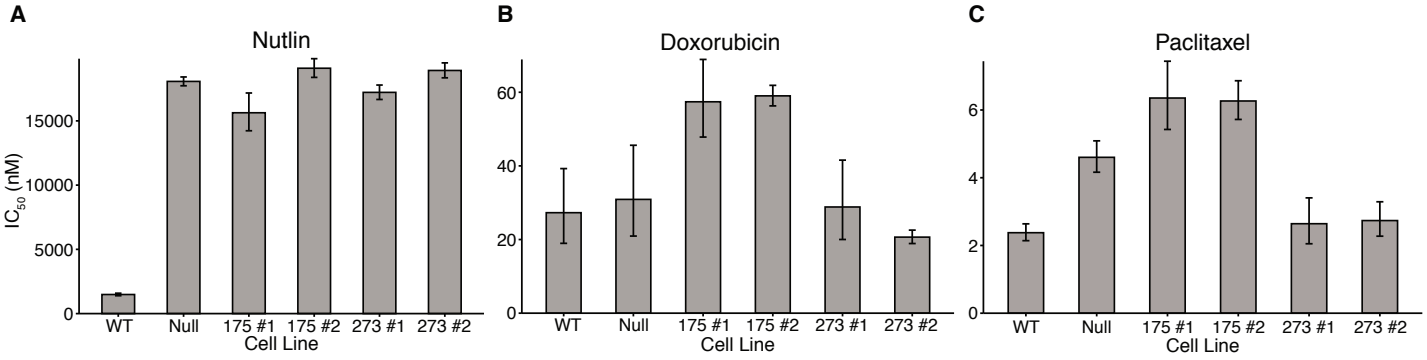

Figure 2. Isogenic p53 GOF mutants confer differential chemoresistance. Isogenic cell lines expressing homozygous wild-type (WT), homozygous frameshift null (Null), heterozygous R175H/frameshift null (175 #1-2), or heterozygous R273H/frameshift null (273 #1-2) p53 were treated with the indicated agents for 72 hours to determine half-maximal inhibitory concentrations, as depicted on the Y axis. Viability was determined by Alamar Blue assay (Invitrogen) and normalized to untreated controls. Error bars depict the 95% confidence intervals of two experimental replicates consisting of four technical replicates each.

Specific Aim 2: To avoid expending valuable time and resources on targets for which there is a low probability of success for therapeutic intervention, we will clone, express, and purify potential target proteins (identified and validated through synthetic lethal screening in Aim 1) and screen them against a molecular fragment library (~15,000 compounds, <300 M.W.) using two-dimensional heteronuclear single quantum correlation (HSQC) NMR (of uniformly ^{15}N-labeled proteins) or saturation transfer difference (STD) NMR (of unlabeled proteins). Proteins that exhibit binding to >0.1% of the molecules in this screen will be considered druggable and will be candidates for further discovery efforts including modifying the hits obtained in fragment-based screens to produce molecules that bind more tightly to the target protein. These efforts will be guided by NMR and/or X-ray crystal structures of protein-ligand complexes using iterative structure-based design. Compounds will also be optimized for their ability to block the biochemical and cellular functions of the target, leading to an inhibition of growth of cancer cell lines.

As noted in the previous reporting period, we are prepared to begin the experiments outlined above upon the completion of the biochemical characterization and genetic screening proposed in Aim 1.

Training and Professional Development Provided by the Project to Date

This IDEA award has promoted the training and career development of Timothy Shaver, a very talented pre-doctoral fellow in the laboratory. Tim's current dissertation work lies at the intersection of two long-standing areas of investigation in the laboratory: the molecular biology of the p53 tumor suppressive response and the identification of therapeutic targets in cancer. A major focus of his dissertation research is determining the mechanism and "targetability" of a mutant p53-adapted state in triple-negative breast cancer. Tim's preliminary results led to the successful submission for this Department of Defense Breast Cancer Research Program IDEA Award. In addition, on the strength of this research and his record to date, he received an F31 predoctoral NRSA fellowship last year. Thus, this IDEA award supports the methods and supplies for the experiments outlined as well as covers the effort and provides the active training of a senior research associate, Hailing Jin.

Based on Tim's progress with the research aims of this award, he attended the International p53 Workshop in Stockholm last summer and presented an abstract and this coming fall will attend the 8th International MDM2 Workshop in New Orleans, Louisiana. The meeting will be held at Tulane University and feature an array of international experts in the p53 field to whom Tim will gain access and with whom he will enjoy scientific discussion and interactions. As was the case at the prior international meeting, Tim will present an abstract of his work (reported below). Due to the small and focused nature of the meeting, Tim will have the opportunity to discuss this research one-on-one and network with principal investigators and trainees in this exciting field of study.

Dissemination of Results to Communities of Interest

Poster Presentation in fall of 2015

Shaver TM, Jin H, Tang L, and Pietenpol JA. "Targeting the p53 mutant-adapted state in triple-negative breast cancer." 8th International MDM2 Workshop, New Orleans, LA, November 2015.

Plans for Next Reporting Period to Accomplish the Goals

Specific Aim 1: We will continue our characterization of our isogenic p53 mutant cell line panel. To take advantage of the cell context-identical nature of this TNBC cell line-derived panel, we will conduct transcriptional assays, co-immunoprecipitation studies, and metabolic analysis to identify pathways that are critical to the p53 LOF and GOF-adapted states. We will additionally proceed with the siRNA screen outlined in our original proposal, using both commercial cell lines and our novel isogenic cell line panel to independently validate genes whose knockdown specifically impairs mutant cells. We anticipate completing these experiments within the first half of Year 3 of the award period.

Specific Aim 2: As noted in the previous annual report, the most promising candidates from each sub-aim of Specific Aim 1 (LOF vs. GOF adaptive states) will be advanced to small molecule screening to evaluate their potential for druggability and to identify promising lead molecules for pharmaceutical development. Using a "fragment library" of 10,000 small molecules with a molecular weight of 280 or less, we will conduct NMR screens to assess protein-ligand interactions. Proteins with hit rates >0.1% will be undergo a fragment-based drug design protocol, as outlined in our original proposal, in addition to screening with more traditional chemical libraries. Lead optimization and cellular evaluation will be conducted on an ongoing basis once candidate protein-ligand partners are

identified. We anticipate completing our identification of screening candidates within the first half of Year 3 of the award period, with ongoing optimization and evaluation throughout the remainder of the award period.

In addition, based on the results presented in Figure 2, we are in the process of assessing a broader array of established and investigational pharmaceutical inhibitors, with the goal of determining differential sensitivity in the GOF and LOF p53 mutant states at large and between individual GOF mutants. Where available, we will compare our results to clinical trial data where p53 mutant status is available.

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4. IMPACT -- KEY RESEARCH ACCOMPLISHMENTS

- Generation of over 16 isogenic clones expressing three of the highest-frequency p53 GOF mutants found in TNBC, along with wild-type and LOF controls. Once we have fully characterized these models and results are peer-reviewed, these reagents will be available to the scientific community.

- Determination of half-maximal inhibitory concentration (IC_{50}) of several standard-of-care chemotherapeutic agents used in TNBC along with Nutlin-3, an MDM2/p53 binding inhibitor across the panel of isogenic clones relative to parental controls. Once we have completed the analyses for a larger panel of drugs and the results are peer-reviewed, these data will be available to the scientific community.

5. CHANGES/PROBLEMS

Nothing to report at this time

6. PRODUCTS

Publications and Presentations

Poster Presentation (Abstract Accepted):

Shaver TM, Jin H, Tang L, and Pietenpol JA. “Targeting the p53 mutant-adapted state in triple-negative breast cancer.” 8th International MDM2 Workshop, New Orleans, LA, November 2015.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Nothing to report at this time.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report at this time.

9. APPENDICES

Nothing to report at this time.